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A novel toxin–antitoxin operon *talA/B* from the Gram-positive bacterium *Leifsonia xyli* subsp. *cynodontis*

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Abstract Here we report a toxin–antitoxin (TA) operon *talAB* identified from the Gram-positive bacterium *Leifsonia xyli* subsp. *cynodontis*. It is shown that *talB* encodes a broad-host cytotoxin functioning in different Gram-positive bacteria, while *talA* encodes its antidote. TalA and TalB form different hetero-oligomers in vitro; these hetero-oligomers, but not the antitoxin TalA, strongly bind to the *talAB* promoter region containing two inverted repeats. This represents a new mechanism of binding the promoter of a TA operon by the toxin and antitoxin complexes.

Keywords: *talA/B*; Toxin–antitoxin; Promoter binding; *Leifsonia xyli* subsp. *Cynodontis*

1. Introduction

Toxin–antitoxin (TA) loci are abundant in the plasmids and chromosomes of some Gram-negative, Gram-positive bacteria and archaea. Most TA systems consist of a pair of genes organized in an operon, of which the downstream gene encodes a stable toxin and the upstream gene encodes an unstable antitoxin. The antitoxin neutralizes the effect of the toxin by directly binding with it [1].

All reported TA operons are autoregulated at the level of transcription by binding of antitoxins to the TA promoter regions [1]. Antitoxins use different motifs to achieve their binding capability such as ribbon-helix-helix motif [2], looped-hinge-helix (LHH) fold [3], helix-turn-helix (HTH) motif [1] and some novel protein folds [4]. The antitoxin binding represses the operon transcription, and the toxin functions as a corepressor to enhance antitoxin binding [5].

Most of the well-characterized TA systems reside in Gram-negative bacteria. Recently, some TA loci from Gram-positive bacteria have been studied, such as *higBA* loci in the *Vibrio cholerae superintegron* [6], *axe–txe* in *Enterococcus faecium* [7], *relBE2* in *Streptococcus pneumoniae* [8], ω–ε–ξ in *Streptococcus pyogenes* [9] and *ydcDE* operon in *Bacillus subtilis* [10], but the molecular mechanisms of TA systems in Gram-positive bacteria are still obscure.

Leifsonia xyli subsp. *cynodontis* (*Lxc*) is an endophytic bacterium originally isolated from Bermuda grass [11], which may serve as an engineered bacterium to improve the crop yield and quality [12]. Interestingly, during the course of studying the insertion sequence of *Lxc*, we have previously identified a truncated TA cassette fused with a partial segment of IS *1237* in pCXC100 [13].

In this study, a complete copy of this TA cassette from the chromosome of *Lxc* was identified; this TA cassette is organized as an operon named as *talA/B* (TA of *Lxc*). The downstream TalB displayed toxicity in *Lxc* and two other Gram-positive bacteria. Disparate from what is previously reported, we found that the TalA–TalB hetero-oligomers, but not the antitoxin TalA, bind to the *talAB* promoter region. This represents a new mechanism of regulating a TA operon transcription by the toxin and antitoxin complexes.

2. Materials and methods

2.1. Plasmids, bacterial strains, media and culture conditions

All bacterial strains and plasmids constructed and used in this study are listed in Supplementary Table S2. *Escherichia coli*, *B. subtilis* and *Corynebacterium glutamicum* were grown in Luria–Bertani (LB) medium supplemented with appropriate concentrations of antibiotics when necessary. *Lxc* were grown in DM medium [12] supplemented with 2 μg/ml tetracycline (Tc) when necessary.

2.2. Protein purification

The TalA-His_6 antitoxin and the TalB-His_6 toxin were purified by Ni^{2+} affinity column according to the manufacture's instructions (QIAGEN). The TalA-His_6 released from the column was loaded onto a DEAE-Sepharose fast flow column (Pharmacia), and eluted using a NaCl gradient. The TalB-His_6 released from the Ni^{2+} affinity column was dialyzed in low-salt solution (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.5 mM EDTA). The TalB sediment was dissolved into 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 0.5 mM EDTA and 10% glycerol before use. The TalA-His_6 was diluted in 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.5 mM EDTA and 10% glycerol. The concentrations of these two proteins were determined by BCA™ Protein Assay Kit (PIERCE).

2.3. Toxicity and neutralization assays

The toxicity of TalB in *Lxc* was tested by electrotransformation of 8 ng of plasmids pLC011, pLC-P3-talB and pLC-P3 into this bacterium, followed by counting the *Lxc* colonies on selective medium. The toxicity of TalB was tested in *B. subtilis* by transformation of 24 ng of pBE2, pBE-PS10-talA and pBE-PS10-talB, and in *C. glutamicum* (strain ATCC13032) by transformation of 30 ng of pEC-XC99E, pEC-XC99E-talA and pEC-XC99E-talB. The neutralization effect of TalA was performed by transformation of 10 ng of pECIR-gus, pECIR-talB and pECIR-talB-trc-talA into *C. glutamicum*.

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Abbreviations: TA, toxin–antitoxin; EGS, ethylene glycol-bis

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2.4. Plasmid stability assay

The stability of pREG45 and pREG-TA in *B. subtilis* 168 was determined as follows. Three colonies containing control plasmid pREG45 [7] and three containing pREG-TA were grown overnight 37 °C in LB supplemented with spectinomycin, then 10 μl of overnight cultures were transferred into 10 ml of fresh, antibiotic-free LB. After 12 h (about 10 generations) of growth, the samples were diluted and incubated until 60 generations of growth under non-selective conditions were reached. At every 10-generation interval, dilutions of cultures were plated on non-selective LB agar, and 100 colonies were picked up from each plate for resistance test to spectinomycin.

2.5. Gel retardation assay

A 198 bp PCR product (using Ta20 and Ta21, Supplementary Table S1) of *talA/B* promoter region was end-labeled by T4 polynucleotide kinase with [γ-^{32}P] ATP (Furui Biotech, Beijing, China). The binding reactions (20 μl) were carried out at 30 °C for 30 min with purified proteins, 2 μl of 250 μg/ml poly(dI-dC) and 2 μl of labeled DNA fragment in the binding buffer (50 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM dithiothreitol and 5% glycerol). Electrophoresis was performed in TBE buffer at 300 V in 5% native polyacrylamide gel at 4 °C.

2.6. DNase I footprinting assay

The 198 bp *talA/B* promoter region was cleaved by endonucleases *Hin*dIII, leaving a sticky 5′ end that is four-nucleotide from the original end (Supplementary Table S1). The recessive 3′ end was labeled with [α-^{32}P] dATP (Furui Biotech, Beijing, China) by Klenow fragment, and then subjected to the same binding reaction as in the gel retardation assay. DNase I footprinting was performed as previously described [14]. The ladders were produced using the Sanger dideoxy method and Ta20 primer that was end-labeled by T4 polynucleotide kinase and [γ-^{32}P] ATP. Therefore, the cleavage of samples at a position ran four-nucleotide faster than the ladder stopped at the same position.

2.7. Ethylene glycol-bis (EGS) cross-linking assay

The experiment was performed according to the protocol of EGS (PIERCE). The samples were loaded on a denatured 15% sodium dodecylsulfate–polyacrylamide gel electrophoresis gel that was stained by SilverSNAP Stain Kit II (PIERCE) after electrophoresis.

3. Results

3.1. Cloning and sequence analysis of the talA/B cassette

The complete copy of the *talA/B* cassette from the chromosome of the plasmid-free *Lxc* strain #3 was obtained by Tail-PCR using D26, D27, D28, D29 primers and AD1, AD2, AD3, AD4 arbitrary degenerate primers [13]. Sequence analysis showed that the *talA/B* cassette is resided in a 759 bp sequence (GenBank Accession Number EF423614). The upstream *talA* and downstream *talB* genes are organized as an operon similar to those of the other TA loci, and the stop codon TGA of the *talA* reading frame overlapped with the GTG start codon of *talB* (Fig. 1A). Protein alignment analysis of known toxin and antitoxin proteins in the database revealed that TalA and TalB proteins are homologous to proteins belonging to RelB/E superfamily (Supplementary Fig. S1).

The putative consensus −35 (5′-GGTAG-3′) and −10 (5′-TAATAA-3′) boxes are located upstream of the ATG site of *talA* (Fig. 1B). Two pairs of inverted repeats (IR) were found, with the 6-bp perfect IR1s surrounding −10 region and the 10-bp near perfect IR2s surrounding −35 region. These IRs could be the potential binding sites for transcriptional regulators (Fig. 1B), as that has been suggested for the *relBE2* in *S. pneumoniae* [8].

3.2. talA/B is a functional TA cassette in Gram-positive bacteria

The toxicity of the predicted toxin protein TalB in its native host *Lxc* was tested by electrotransformation of the empty plasmid (pLC011), the plasmid containing *talB* gene driven by a strong promoter (pLC-P3-talB) and the plasmid only containing the promoter (pLC-P3) into this bacterium. The transformation efficiency of pLC011 and pLC-P3 were 2.3×10^4 and 1.0×10^4 μg^{-1}, while no colony was recovered when the TalB expression plasmid pLC-P3-talB was transformed (Table 1).

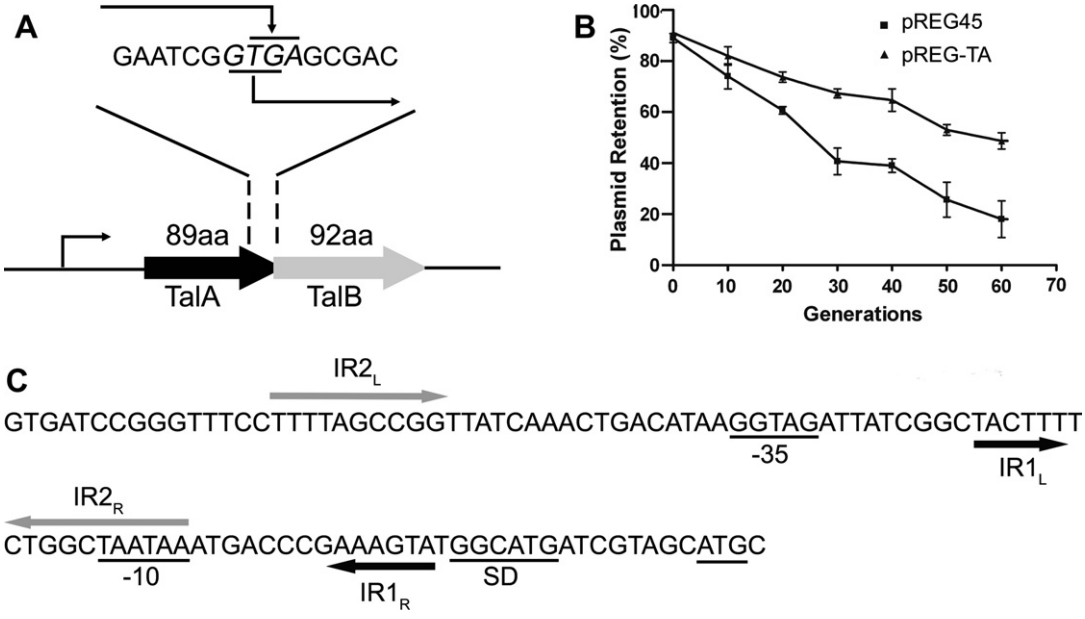

Fig. 1. *talA/B* is a functional toxin–antitoxin cassette. (A) The organization of the *talA/B* operon, the overlapped UGA stop codon for TalA and GUG start codon for TalB are indicated. (B) Characteristic structures in the promoter region. The −10 and −35 regions of the promoter are underlined, and the two pairs of inverted repeats are indicated by arrows. (C) Segregational stability assay of pREG45 (squares) and pREG-TA (triangles) in *B. subtilis* 168.

These results indicated that TalB is a toxin in its high-GC native host *Lxc*. It was also shown that TalB was cytotoxic in two other Gram-positive bacteria including the low-GC *B. subtilis* and medium-GC *C. glutamicum* (Table 1). However, this protein was expressed very well in the Gram-negative bacterium *E. coli* without inhibiting the growth of host, which allowed us to overexpress both the TalB and TalB-His_6 protein to perform biochemical analysis as detailed below. Therefore, we concluded that TalB is a toxin broadly functioning in Gram-positive hosts, but not in the Gram-negative host.

C. glutamicum was then used to study the antitoxin function of TalA (Table 2). We placed the toxin and antitoxin genes under two different promoters. The *talB* gene was cloned into the expression vector pECIR-gus to replace the original *gus* gene. No colony was recovered on the plate after electro-transformation of this recombinant plasmid (pECIR-talB), suggesting that TalB displays a toxin function in *C. glutamicum*. The *talA* gene driven by *trc* promoter was then cloned into pECIR-talB downstream of talB expression cassette to generate a new recombinant plasmid (pECIR-talB-trc-talA) that express both toxin and antitoxin proteins. Strikingly, transformation of this new plasmid into *C. glutamicum* produced a large number of colonies, as did the control plasmid containing no toxin gene. These results are consistent with the conclusion that *talA* is the antitoxin gene encoding a protein counteracting the toxicity of *talB*.

It has been shown that many TA cassettes are able to increase the plasmids stability in bacteria. Because the antitoxins are metabolically unstable, the newborn plasmid-free cells are killed by the stable toxins inherited from mother cells. In this way, TA loci prevent the proliferation of plasmid-free cells in growing bacterial cultures [1]. Interestingly, when it was cloned into a segregational unstable plasmid pREG45, the *talA*/*B* cassette significantly increased the plasmid stability in *B. subtilis* (pREG-TA shown in Fig. 1C). Therefore, the *talA*/*B* operon is a functional segregational stability cassette that partially stabilizes an unstable plasmid.

Table 1
The toxicity assays of TalB in *Lxc*, *B. subtilis* and *C. glutamicum*

Bacteria	Plasmids	Transformation efficiency
Lxc	pLC011	2.3×10^4 μg^{-1}
Lxc	pLC-P3-talB	0
Lxc	pLC-P3	1.0×10^4 μg^{-1}
B. subtilis	pBE2	$2.5 \pm 1.8 \times 10^4$ μg^{-1}
B. subtilis	pBE-PS10-talA	$6.3 \pm 1.2 \times 10^3$ μg^{-1}
B. subtilis	pBE-PS10-talB	0
C. glutamicum	pEC-XC99E	$4.5 \pm 0.8 \times 10^3$ μg^{-1}
C. glutamicum	pEC-XC99E-talA	$4.9 \pm 0.9 \times 10^3$ μg^{-1}
C. glutamicum	pEC-XC99E-talB	0

Table 2
The function of TalA in neutralizing TalB toxicity

Bacteria	Plasmids	Transformation efficiency
C. glutamicum	pECIR-gus	$1.5 \pm 0.3 \times 10^3$ μg^{-1}
C. glutamicum	pECIR-talB	0
C. glutamicum	pECIR-talB-trc-talA	$1.3 \pm 0.3 \times 10^3$ μg^{-1}

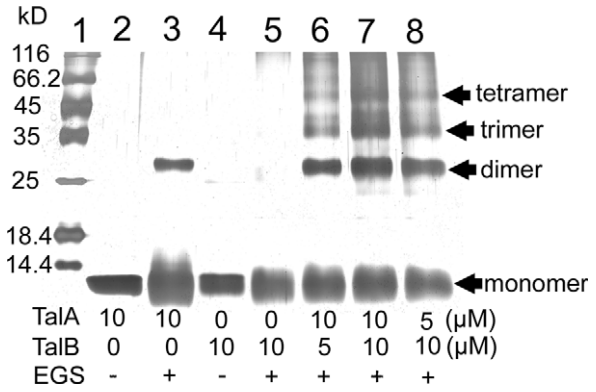

Fig. 2. TalA and TalB form hetero-oligomers. Lane 1 is the protein ladder. The indicated concentrations of TalA and TalB were added in lanes 2–8, EGS was added at 1 mM in each lane, except for lanes 2 and 4.

3.3. TalA and TalB form different hetero-oligomers

Most of the well-studied toxins and antitoxins form homo- and hetero-oligomers [1]. EGS cross-linking experiments were performed to elucidate the homo- and hetero-oligomerization features of TalA and TalB proteins. Fig. 2 shows that a significant fraction of TalA dimerized in the phosphate buffer (pH 8), while TalB could hardly dimerize. When both proteins were present, the large protein complexes spanning from 35 to 40 kDa appeared in a significant amount, indicating the formation of hetero-trimer(s) between TalA and TalB. These hetero-trimers could be TalA–$TalB_2$ (a TalA monomer and a TalB dimer) and $TalA_2$–TalB (a TalA dimer and a TalB monomer). The presence of TalB increased the level of the dimeric protein compared to that only containing TalA, suggesting the formation of TalA–TalB hetero-dimer. Higher forms of hetero-oligomer were also evident; the 50-kDa population may represent the hetero-tetramer, and an even larger one could represent the hetero-hexamer complex.

3.4. TalA–TalB complexes, but not antitoxin TalA alone, effectively binds to the talAB promoter region to form stable complexes

All well studied TA operons are autoregulated at the level of transcription by the binding of antitoxins to the TA promoters. Surprisingly, gel-retardation assays showed that up to 50 μM of antitoxin TalA did not shift the radiolabeled promoter DNA (Fig. 3A), while a very faint shifted band was observed when TalB was added. Interestingly, in the presence of 15 μM of TalB, 0.5 μM TalA shifted a significant fraction of the labeled promoter DNA to a slowly migrating band, and the shifted fraction was increased with higher TalA concentrations. These results strongly suggested that TalA binds to the promoter DNA effectively only when it forms complexes with TalB; this newly formed TalA–TalB–promoter DNA complex was named as the lowershift complex (Fig. 3B, lanes 3–5). Clearly, the lack of the promoter binding ability when TalA was present alone could be due to an unexpected low affinity of TalA with its promoter DNA, instead of the possible disruption of the TalA protein function.

TalA at a concentration of 4.5 μM promoted the formation of a highershift complex presumably containing more TalA and TalB units (Fig. 3B). Moreover, TalA $\geqslant$ 7.5 μM resulted in a more slowly migrating supershift band, indicating the

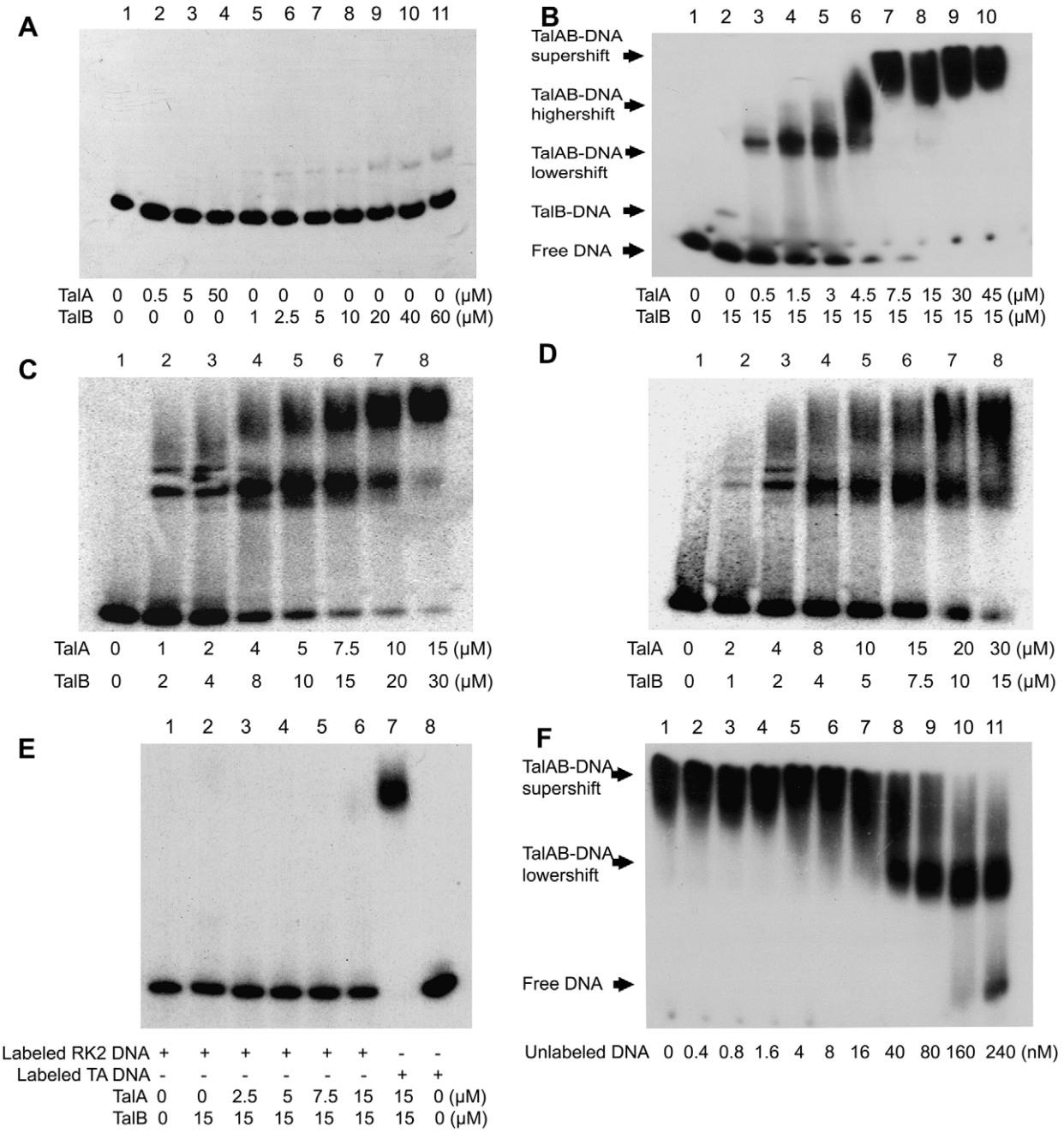

Fig. 3. TalA–TalB complexes bind to the *talAB* promoter. Gel retardation assays were performed to assay the independent binding of TalA or TalB with the *talAB* promoter DNA (A), and the cooperative binding of TalA–TalB complexes with the promoter (B–D). (B) A fixed concentration of TalB was present, and varying concentrations of TalA was added. (C) Varying concentrations of TalA and TalB were added with the 1:2 ratio between TalA:TalB being maintained. (D) Similar as in (C) except for the ratio was changed to 2:1 (TalA:TalB). (E) TalA–TalB complexes do not bind the RK2 plasmid DNA. A 200-bp PCR DNA fragment from RK2 plasmid was end-labeled, and 0.4 nM of the labeled DNA was used in each reaction. The gel retardation experiment was performed similarly as in (B). (F) Competition assay. Each reaction contained 0.4 nM of labeled DNA, 15 μM of TalA and TalB, and the unlabeled DNA fragment at the indicated concentrations was used as competitor.

formation of a very large nucleoprotein complex (Fig. 3B, lanes 7–10). The supershift band could result from the presence of more than one DNA binding sites on the promoter DNA and/or the formation of higher forms of hetero-oligomer among TalA and TalB, as do the other toxin and antitoxin proteins [1].

Fig. 3B suggests that TalA:TalB at a 1:2 ratio converted the lowershift complex to the supershift complex. The results shown in Fig. 3C and D further support the 1:2 ratio between TalA and TalB is important for the efficient formation of the supershift complex. Meanwhile, the adequate concentrations of these two proteins are also critical for the formation of these two different complexes.

It is noticeable that TalA–TalB complex did not bind to the origin of RK2 plasmid (~200 bp), thus supporting the specificity of TalA–TalB binding to their own promoter DNA (Fig. 3E). Competition experiments showed that about 100-fold higher concentration of the unlabeled DNA (40 nM) than

the radiolabeled counterpart started to convert the supershift form of the TalA–TalB–DNA complex to the lowershift form, and a complete conversion was achieved by 400-fold unlabeled DNA. Strikingly, 600-fold higher concentration of the unlabeled DNA (240 nM) was required to convert only a small fraction of the lowershift band to the free DNA band (Fig. 3F). These results suggested that TalA and TalB proteins bind to their promoter DNA very tightly in the supershift and lowershift complexes. On the contrary, the highershift complex was not as distinguishable as the supershift and lowershift complexes, suggesting that it is an unstable intermediate complex.

3.5. TalA–TalB hetero-oligomers bind to IR regions

DNase I footprinting was performed to reveal the binding sites of TalA–TalB complex on the *talAB* promoter DNA. Two regions of the *talAB* promoter DNA were significantly protected when TalA and TalB were present (Fig. 4A). These two regions covered two palindromic sequences, IR1 and IR2, in the promoter DNA (Fig. 1B). Protection at both $IR1_L$ and $IR1_R$ regions, as well as at $IR2_R$ occurred at all TalA concentrations, suggesting that low concentrations of TalA–TalB complex readily bind to these regions. In contrast, the protection at $IR2_L$ was strongly dependent on high TalA concentrations; this protection was expanded and became stronger when TalA reached the concentration of TalB (Fig. 4A). Surprisingly, the DNA sequence between $IR2_L$ and $IR2_R$ became more accessible to DNase I upon the protein binding at $IR2_L$, while the loop between $IR1_L$ and $IR1_R$ was at the protected state at all TalA concentrations. These results suggest a model that the TalA–TalB hetero-oligomer readily occupies the entire IR1 region and $IR2_R$ overlapping with $IR1_L$. Further oligomerization brings $IR2_L$ close to $IR2_R$, and this DNA looping exposes the region between $IR2_L$ and $IR2_R$ (Fig. 4B).

4. Discussion

In this study, we reported a functional TA locus, named as *talA/B*, from a Gram-positive endophytic bacterium *Lxc*. The two composite genes in this locus are organized as an operon, with the downstream gene *talB* encoding a protein exhibiting cellular toxicity in its native host *Lxc*, and in other Gram-positive bacteria *C. glutamicum* and *B. subtilis*, but not in the Gram-negative *E. coli*. The presence of *talA/B* cassette also partially stabilizes a segregational unstable plasmid in *B. subtilis*.

TalA and TalB efficiently form different hetero-oligomers, which bind to the *talAB* promoter region and form two different forms of stable TalA–TalB–DNA complexes. DNase I footprinting of the TalA–TalB binding sites on the promoter suggests that the lowershift complex could result from the binding of a hetero-hexamer to IR1 sites in the promoter, and the supershift complex from the binding of one more hetero-hexamer to IR2 sites (Fig. 4).

Surprisingly, unlike all the other well-studied TA operon, antitoxin TalA at up to 50 μM displays no promoter binding activity. Consistently, the secondary structure prediction reveals no typical DNA binding motif in TalA, instead, a ribbon-helix-helix DNA binding motif is found at the N-terminal region of TalB. Consistent with the result that TalA has a strong dimerization ability, TalA harbors four predicted α-helices, and three of them are rich in hydrophobic residues that are repeated every three to four residues (Supplementary

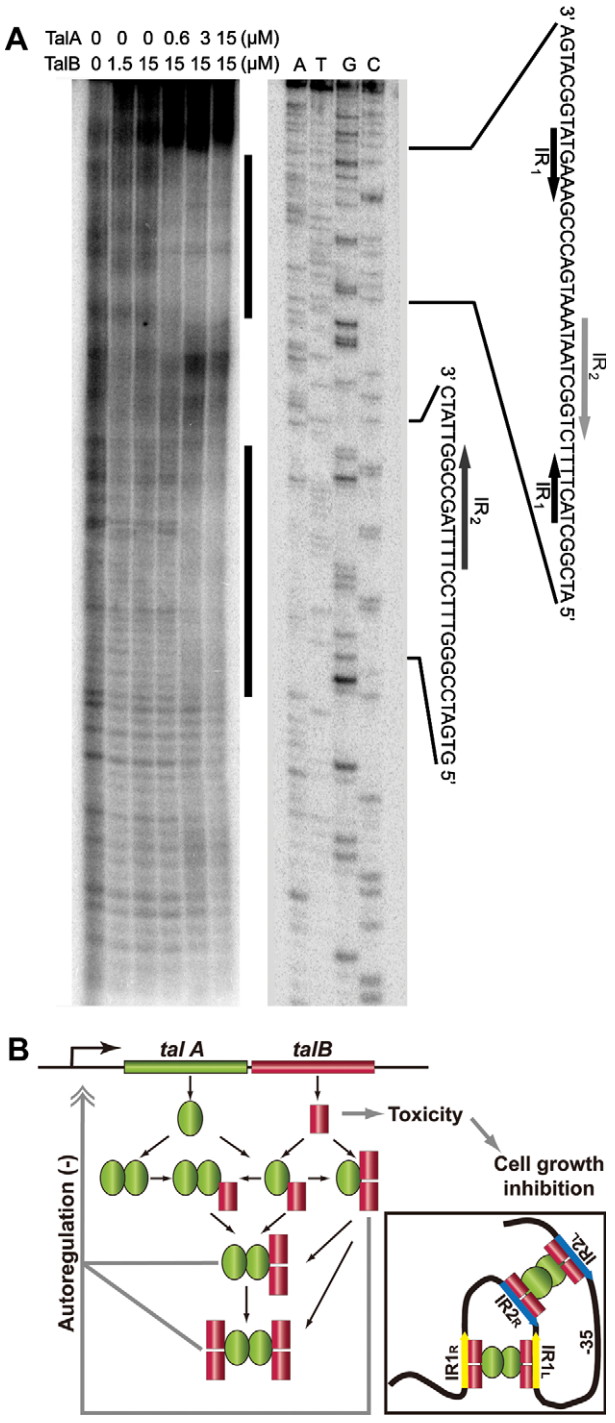

Fig. 4. TalA–TalB complexes bind to the promoter regions containing two inverted repeats. (A) Footprinting assay of the protection of *talAB* promoter against DNase I digestion by the TalA–TalB complex binding (left panel). The ladders were shown at the right panel with the protected nucleotide sequences being listed. The four segments of the two inverted repeats were indicated. (B). Schematic model of the transcription autoregulation of the *talAB operon*. The *talA* gene and TalA protein are shown in green and the *talB* gene and TalB protein in red. Each protein complex is represented by an appropriate combination of filled green ellipses (TalA) and red rectangles (TalB).

Fig. S2). The crystal structure of the archaeal RelE–RelB complex has revealed that the antitoxin RelB dimerizes through its hydrophobic surfaces [15]. In contrast, only one α-helix containing a hydrophobic surface was predicted for TalB, which could explain the poor dimerization capability of TalB (Supplementary Fig. S2). Therefore, we propose that TalA not only forms homodimer, but also provides dimerization surfaces for TalB, which promotes the formation of TalA–$TalB_2$ trimer and $TalB_2$–$TalA_2$–$TalB_2$ hexamer that is capable of binding to the inverted repeats in the promoter by the RHH motif of TalB (Fig. 4). This model is consistent with our finding that 1:2 ratio between TalA and TalB is critical for efficient formation of the supershift complex. The antitoxin MazE has been shown to interact with two MazF toxin molecules, although using a different mechanism [16]. Therefore, although different TalA–TalB oligomers are formed, we propose only those containing a TalA–$TalB_2$ trimer unit binds to DNA. This hypothesis is supported by a recent study showing that the hetero-oligomers of Kid and Kis proteins, the toxin and antitoxin encoded by the *parD* operon of *E. coli* plasmid R1, differ in their DNA binding activity [17]. It is also possible that the presence of promoter DNA could promote further oligomerization when multiple binding sites are present. Further study is required to address this question.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.08.012.

References

[1] Gerdes, K., Christensen, S.K. and Løbner-Olesen, A. (2005) Prokaryotic toxin–antitoxin stress response loci. Nat. Rev. Microbiol. 3, 371–382, (Review).
[2] Anantharaman, V. and Aravind, L. (2003) New connections in the prokaryotic toxin–antitoxin network: relationship with the eukaryotic nonsense-mediated RNA decay system. Genome Biol., R81.
[3] Vaughn, J.L., Feher, V., Naylor, S., Strauch, M.A. and Cavanagh, J. (2000) Novel DNA binding domain and genetic regulation model of *Bacillus subtilis* transition state regulator *abrB*. Nat. Struct. Biol. 7, 1139–1146.
[4] Cherny, I. and Gazit, E. (2003) The YefM antitoxin defines a family of natively unfolded proteins: implications as a novel antibacterial target. J. Biol. Chem. 279, 8252–8261.
[5] Gotfredsen, M. and Gerdes, K. (1998) The *Escherichia coli* relBE genes belong to a new toxin–antitoxin gene family. Mol. Microbiol. 29, 1065–1076.
[6] Christensen-Dalsgaard, M. and Gerdes, K. (2006) Two *higBA* loci in the *Vibrio cholerae* superintegron encode mRNA cleaving enzymes and can stabilize plasmids. Mol. Microbiol. 62, 397–411.
[7] Grady, R. and Hayes, F. (2003) Axe–Txe, a broad-spectrum proteic toxin–antitoxin system specified by a multidrug-resistant, clinical isolate of *Enterococcus faecium*. Mol. Microbiol. 47, 1419–1432.
[8] Nieto, C., Pellicer, T., Balsa, D., Christensen, S.K., Gerdes, K. and Espinosa, M. (2006) The chromosomal *relBE2* toxin–antitoxin locus of *Streptococcus pneumoniae*: characterization and use of a bioluminescence resonance energy transfer assay to detect toxin–antitoxin interaction. Mol. Microbiol. 59, 1280–1296.
[9] Zielenkiewicz, U. and Ceglowski, P. (2005) The toxin–antitoxin system of the streptococcal plasmid pSM19035. J. Bacteriol. 187, 6094–6105.
[10] Pellegrini, O., Mathy, N., Gogos, A., Shapiro, L. and Condon, C. (2005) The *Bacillus subtilis ydcDE* operon encodes an endoribonuclease of the MazF/PemK family and its inhibitor. Mol. Microbiol. 56, 1139–1148.
[11] Evtushenko, L.I., Dorofeeva, L.V., Subbotin, S.A., Cole, J.R. and Tiedje, J.M. (2000) *Leifsonia poae gen.* nov., sp. nov., isolated from nematode galls on Poa annua, and reclassification of '*Corynebacterium aquaticum*' Leifson 1962 as *Leifsonia aquatica* (ex Leifson 1962) gen. nov., nom. rev., comb. nov. and *Clavibacter xyli* Davis et al., 1984 with two subspecies as *Leifsonia xyli* (Davis et al., 1984) gen. nov., comb. nov.. Int. J. Syst. Evol. Microbiol. 50, 371–380.
[12] Li, T.Y., Zeng, H.L., Ying, P., Lin, H., Fan, X.L., Guo, Z.G. and Zhang, C.F. (2007) Construction of a stable expression vector for *Leifsonia xyli* subsp. *cynodontis* and its application in studying the effect of the bacterium as an endophytic bacterium in rice. FEMS Microbiol. Lett. 267, 176–183.
[13] Lin, H., Li, T.Y. and Zhang, Y. (2007) Characterization of the variants, flanking genes and promoter activity of *Leifsonia xyli* subsp. *cynodontis* insertion sequence IS1237. J. Bacteriol. 89, 3217–3227.
[14] Yin, P., Li, T.Y., Xie, M.H., Jiang, L. and Zhang, Y. (2006) A Type Ib ParB protein involved in plasmid partitioning in a gram-positive bacterium. J. Bacteriol. 188, 8103–8108.
[15] Takagi, H., Kakuta, Y., Okada, T., Yao, M., Tanaka, I. and Kimura, M. (2005) Crystal structure of archaeal toxin–antitoxin RelE–RelB complex with implications for toxin activity and antitoxin effects. Nat. Struct. Mol. Biol. 12, 327–331.
[16] Kamada, K., Hanaoka, F. and Burley, S.K. (2003) Crystal structure of the MazE/MazF complex: molecular bases of antidote-toxin recognition. Mol. Cell 11, 875–884.
[17] Monti, M.C., Hernandez-Arriaga, A.M., Kamphuis, M.B., Lopez-Villarejo, J., Heck, A.J., Boelens, R., et al. (2007) Interactions of Kid–Kis toxin–antitoxin complexes with the parD operator–promoter region of plasmid R1 are piloted by the Kis antitoxin and tuned by the stoichiometry of Kid–Kis oligomers. Nucleic Acids Res. 35, 1737–1749.